Observational Study

Medicine

OPEN

Network pharmacology-based strategy to investigate pharmacological mechanism of Liuwei Dihuang Pill against postmenopausal osteoporosis

Qingchan Wang, MM[a], Ping Huang, PhD[b], Chenjie Xia, PhD[c], Danqing Fu, PhD[d,*]

Abstract

Postmenopausal osteoporosis (PMOP) has became 1 of most prevalent bone disorders with aging population. Liuwei Dihuang (LWDH) Pill, a classical kidney-tonifying prescription, is extensively used to treat PMOP in China. The aim of this study is to explore the pharmacological mechanisms of LWDH Pill against PMOP via network pharmacological strategy. The active ingredients of LWDH Pill were screened out from the Traditional Chinese Medicine System Pharmacology, Encyclopedia of Traditional Chinese Medicine and Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine Databases, and their related target genes were fished in the UniProt database. Simultaneously, the GeneCards and DisGeNET databases were used to identify the target genes of PMOP. Through establishing a protein-protein interaction network, the overlapping genes between LWDH Pill and PMOP were identified to analyze their interactions and the hub target genes. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses were performed to predict the underlying biological processes (BP) and signaling pathways, respectively. A total of 64 active ingredients and 653 related target genes were identified in LWDH Pill, and 292 target genes were closely associated with PMOP. After matching the target genes between LWDH Pill and PMOP, 84 overlapping targets were obtained and considered as therapeutically relevant. Through construction of a protein-protein interaction network, we identified 20 hub target genes including IL6, INS, tumor necrosis factor, AKT1, vascular endothelial growth factor A, IGF1, TP53, IL1B, MMP9, JUN, LEP, CTNNB1, EGF, PTGS2, PPARG, CXCL8, IL10, CCL2, FOS and ESR1. Gene Ontology enrichment analysis suggested that LWDH Pill exerted anti-PMOP effects via regulating multiple BP including cell proliferation and apoptosis, oxidative stress, inflammation and angiogenesis. Kyoto Encyclopedia of Genes and Genomes enrichment analysis revealed several pathways, such as PI3K-AKT pathway, mitogen-activated protein kinase pathway, hypoxia-inducible factors-1 pathway, tumor necrosis factor pathway, interleukin-17 (IL-17) pathway and FoxO pathway that might be involved in modulating the above BP. Through network pharmacological approach, we investigated the potential therapeutic mechanism of LWDH Pill against postmenopausal osteoporosis in a systemic perspective. These identified multi-targets and multi-pathways provide promising directions for further revealing more exact mechanisms.

Abbreviations: BATMAN-TCM = Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine, BP = biological processes, DL = drug-likeness, EGF = epidermal growth factor, ETCM = Encyclopedia of Traditional Chinese Medicine, HIF-1 = hypoxia-inducible factors-1, IL-1 = interleukin-1, IL-17 = interleukin-17, IL-6 = interleukin-6, KEGG = Kyoto encyclopedia of genes and genomes, LWDH = Liuwei Dihuang, MAPK = mitogen-activated protein kinase, MDP = Mu Dan Pi, GO = gene ontology, OB = Oral bioavailability, OVX = ovarietomized, PMOP = postmenopausal osteoporosis, PPI = protein-protein interaction, SDH = Shu Di Huang, SY = Shan Yao, SZY = Shan Zhu Yu, TCM = Traditional Chinese medicine, TCMSP = Traditional Chinese Medicine System Pharmacology, TNF = tumor necrosis factor, VEGFA = vascular endothelial growth factor A, ZX = Ze Xie.

Keywords: Liuwei Dihuang pill, network pharmacology, pharmacological mechanism, postmenopausal osteoporosis, traditional Chinese medicine

The study was supported by the National Natural Science Foundation of China (82104885); Natural Science Foundation of Zhejiang Province (Q22H271191, LY18H290007); Traditional Chinese Medical Administration of Zhejiang Province (2021ZQ082); Research Project of Zhejiang Chinese Medical University (2020ZG04, 2020ZR06).

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

The authors have no ethics statement to disclose.

[a] Department of Gynaecology and Obstetrics, The Second People's Hospital of Luqiao District, Taizhou, China, [b] The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China, [c] Department of Orthopedic Surgery, Ningbo University of Lihuili Hospital, Ningbo, China, [d] School of Basic Medical Sciences, Zhejiang Chinese Medical University, Hangzhou, China.

** Correspondence: Danqing Fu, School of Basic Medical Sciences, Zhejiang Chinese Medical University, No. 548, Binwen Road, Hangzhou, Zhejiang Province 310053, China (e-mail: 20191029@zcmu.edu.cn).*

How to cite this article: Wang Q, Huang P, Xia C, Fu D. Network pharmacology-based strategy to investigate pharmacological mechanism of Liuwei Dihuang Pill against postmenopausal osteoporosis. Medicine 2022;101:47(e31387).

Received: 31 January 2022 / Received in final form: 26 September 2022 / Accepted: 28 September 2022

http://dx.doi.org/10.1097/MD.0000000000031387

1. Introduction

Postmenopausal osteoporosis (PMOP), 1 of most prevalent bone disorders, is characterized by low bone mineral density, microstructure deterioration and higher risk of fragility fracture.[1] It is estimated that 15% of postmenopausal women more than 50 years old are suffering in PMOP worldwide, which causes a huge medical and economic burden.[2] Currently, there are a variety of anti-osteoporosis drugs, such as estrogen receptor modulators, parathyroid hormone, bisphosphonates and active vitamin D, are applied to improve bone mineral density and alleviate symptoms.[3] However, most of them have varying side effects that limit the clinical application and efficacy.[4] Therefore, developing a safe and alternative treatment is largely needed.

Traditional Chinese medicine (TCM), mainly based on natural herbs, has attracted extensive attention due to its satisfactory curative effects and relative safety.[5] Liuwei Dihuang (LWDH) Pill, a classical prescription of tonifying kidney, was initially recorded in Song Dynasty (960-1279 AD). It consists of 6 herbs and have been used to treat PMOP for a long time in China.[6,7] Recent studies demonstrated LWDH Pill could attenuate bone loss in ovariectomized (OVX) animal models,[8,9] confirming its anti-PMOP effects. The theory of "kidney governing bones" can well account for its anti-PMOP effects from TCM perspective. Bone loss in PMOP is due to kidney deficiency and LWDH Pill can tonify kidney to strengthen bone.[6,10] Nevertheless, the exact pharmacological mechanism of LWDH Pill against PMOP is still unknown.

For the multi-components and multi-targets of TCM prescription, conventional animal or cellular research strategies can not meet the requirements to study massive gene targets simultaneously.[11] Network pharmacology is an emerging discipline that integrates pharmacology, system biology, bioinformatics, and computer science.[11–13] According to the study pattern of "Medicine – Target – Gene – Disease," network pharmacology provides an integrative and systematic viewpoint to investigate the pharmacological mechanism of LWDH Pill.[13,14]

Here, through network pharmacology, we comprehensively reviewed the potential therapeutic targets of LWDH Pill in treating PMOP. These targets were further performed with bioinformatic analyses to determinate the key therapeutic target genes, biological processes (BP) and signaling pathways. The protocol of our experimental procedures were shown in Figure 1.

2. Materials and methods

Ethical approval was waived or not necessary, all procedures performed in studies do not involve human participants or animals.

2.1. Identification of active ingredients

LWDH Pill is composed of 6 herbs including Shu Di Huang (SDH), Shan Zhu Yu (SZY), Shan Yao (SY), Mu Dan Pi (MDP), Fu Ling and Ze Xie (ZX) (Table 1). The herb names were inputted into Traditional Chinese Medicine System Pharmacology (TCMSP, http://lsp.nwu.edu.cn/tcmsp.php), Encyclopedia of Traditional Chinese Medicine (ETCM, http://www.tcmip.cn/ETCM/) and a Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine (BATMAN-TCM, http://bionet.ncpsb.org.cn/batman-tcm/) to obtain the chemical ingredients. Oral bioavailability (OB) is a pharmacokinetic parameter that reflects the efficiency of drug delivery to the systemic circulation. Drug-likeness (DL) is used to assess drug properties including solubility and chemical stability. Oral bioavailability (OB ≥ 30%) and drug-like (DL ≥ 0.18) were set as the screening criteria,[15] and a total of 64 active ingredients were identified in LWDH Pill.

2.2. Target prediction of LWDH pill

Then, we predicted the targets related to the active ingredients from TCMSP database through using the computer targeting technology developed by the TCMSP. The information of targets was further inputted into UniProt database (https://www.uniprot.org/) to obtain their gene symbols with the filter of "swiss-prot reviewed" and "Homo sapiens." The target genes related to the active ingredients from BATMAN-TCM database and ETCM database were directly obtained from respective databases.

2.3. Collection of PMOP-related genes

The "postmenopausal osteoporosis" was used as the keywords to retrieve disease-related genes in the GeneCards Database (https://www.genecards.org/) and DisGeNET Database (https://www.disgenet.org/). After filtered by score > 0.1 in DisGeNET and score > 10 in GeneCards,[14] a total of 292 PMOP-related genes were finally obtained.

2.4. Venn diagram

A Venn diagram was constructed on an online website (https://bioinfogp.cnb.csic.es/tools/venny/index.html) to identify the overlapping genes between PMOP and LWDH Pill.[11]

2.5. Protein-protein interaction (PPI)

The overlapping genes were imported into the STRING 11.0 (https:// string-db.org/) for PPI analysis with the parameters of "confidence score > 0.4" and "Homo sapiens." The node-node data were exported from STRING and their interconnection network were visualized and analyzed by using Cytoscape 3.7.1. Degree Centrality, Betweenness Centrality and Closeness Centrality, 3 topological properties of the network, were analyzed using plug-in "Network Analysis."[11]

2.6. Gene ontology (GO) enrichment analysis

The overlapping genes were imported into the Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/home.jsp, version 6.8) for GO enrichment analysis.[16] Three sub-items including molecular function, cellular component and biological process (BP) were all analyzed. After filtered by FDR < 0.01, the information of top 40 BP terms were listed in a bubble diagram based on the ascending order of log *P*-value.[11]

2.7. KEGG enrichment analysis

The ID numbers of overlapping genes were imported into Kyoto Encyclopedia of Genes and Genomes (https://www.kegg.jp/) for pathway enrichment analysis. The top 32 signaling pathways were listed in a bar diagram according to the descending order of gene number enriched in each pathway.

2.8. Network construction

We constructed networks as follows: Herb-Ingredient network. Targets-Pathways network. All visualized graphs of above networks were established by Cytoscape3.8.0 software (https://cytoscape.org/index.html).

3. Results

3.1. Active ingredients of LWDH pill

After screening herb names in the TCMSP databases with the thresholds of OB ≧ 30% and DL ≧ 0.18, a total of 64 active ingredients were identified in LWDH Pill, including 20

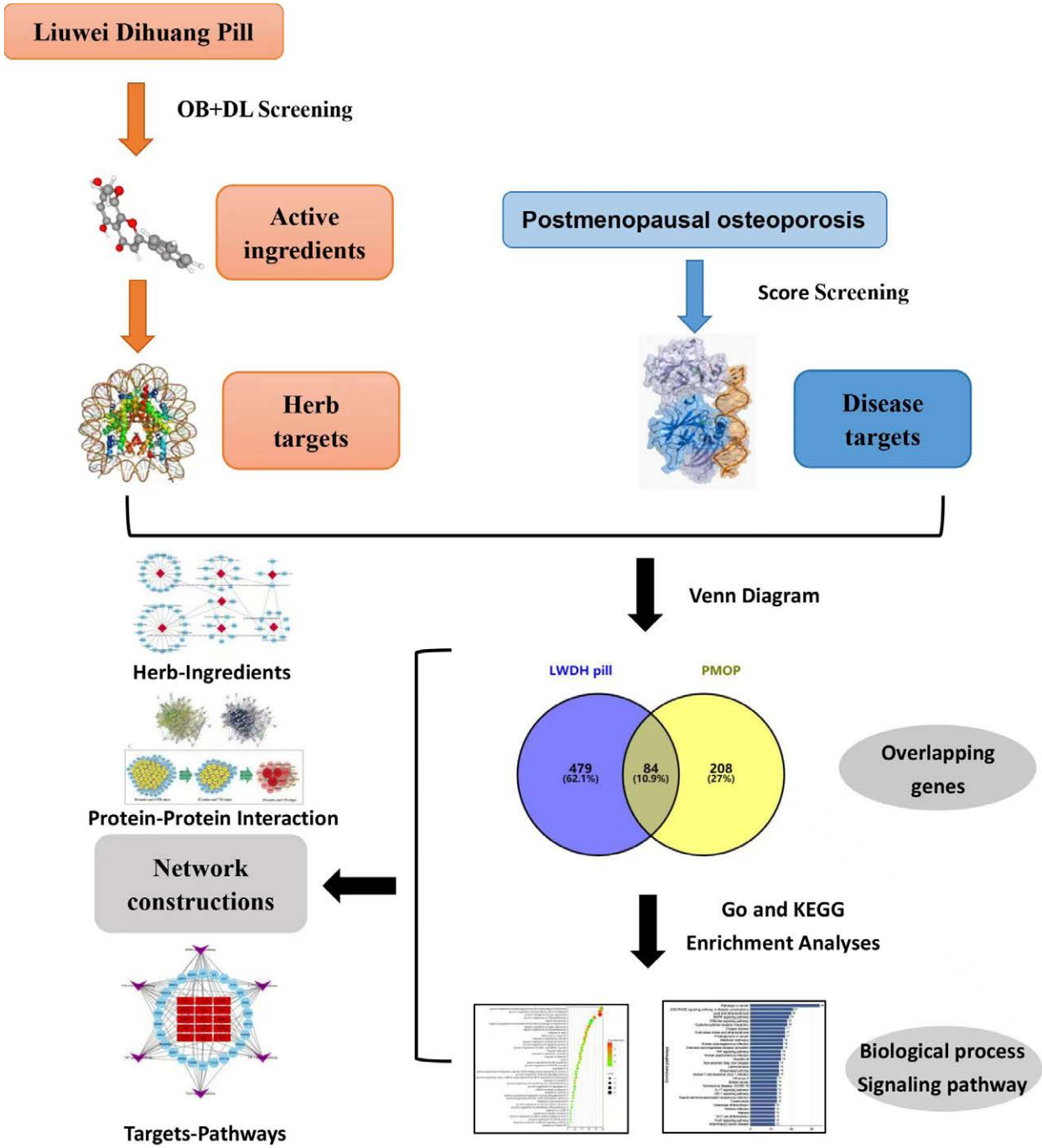

Figure 1. The flow chart of network pharmacology based study for investigating mechanism of LWDH Pill against PMOP. LWDH = Liuwei Dihuang, PMOP = postmenopausal osteoporosis.

ingredients in SZY, 16 in SY, 14 in MDP, 12 in FL, 8 in ZX and 2 in SDH. Then, we constructed a Herb-Ingredient network to visually present the relationship between the herbs and the active ingredients. As shown in Figure 2, beta-sitosterol (DL = 0.75, OB = 36.91) showed the highest frequence, and was contained in SZY, SY, ZX, MDP and SDH; stigmasterol (DL = 0.76, OB = 43.83) listed at second place was found in SZY, SY and SDH.

3.2. Target prediction and analysis

Then, we fished a total of 653 target genes on the 64 related ingredients using the Uniprot database, BATMAN-TCM database and ETCM database. Simultaneously, we integrated the PMOP related genes from GeneCards and DisGeNet databases, and 292 target genes were obtained. After the establishment of Venn diagram, 84 overlapping genes were identified between LWDH Pill and PMOP (Fig. 3).

3.3. PPI network and hub therapeutic targets

Next, these 84 overlapping genes were imported into the String database to build a PPI network, 84 nodes and 1458 edges were obtained. We plotted the PPI network according to the strength of evidence and confidence level (Fig. 4A and 4B), respectively. "Degree Centrality" and "Closeness Centrality" represent the

Table 1

The compositions of LWDH Pill.

Chinese name (Abbreviation)	Botanical name	Common name	Parts	Proportion
Shu Di Huang (SDH)	*Rehmannia glutinos (Gaertn.)*	Rehmannia	Root	32%
Shan Zhu Yu (SZY)	*Cornus officinalis(Sieb.)*	Medical Dogwood	Fruit	16%
Mu Dan Pi (MDP)	*Paeonia suffruticosa (Andr)*	Tree Peony Bark	Root	12%
Shan Yao (SY)	*Dioscoreae opposite (Thunb.)*	Common Yam	Root	16%
Fu Ling (FL)	*Poria cocos (Schw.)*	Tuckahoe	Sclerotia	12%
Ze Xie (ZX)	*Alisma orientale (Sam.)*	Oriental Waterplantain Rhizome	Tuber	12%

LWDH = Liuwei Dihuang.

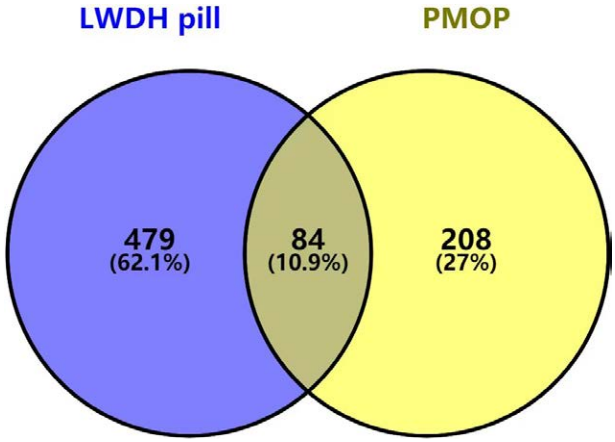

Figure 3. The Venn diagram of the target genes between LWDH Pill and PMOP. The overlap part indicates the underlying therapeutic targets through which LWDH Pill treats PMOP. LWDH = Liuwei Dihuang, PMOP = postmenopausal osteoporosis.

number of edge and the mean distance between target node and others, respectively.[17,18] "Betweenness Centrality" is used to measure code centrality based on the shortest paths.[19] These 3 parameters were used to select the pivot nodes. After the first screening round of Degree ≥ 20, Betweenness ≥ 0.003 and Closeness ≥ 0.57, 42 nodes and 710 edges were obtained. Through the second screening round of Degree ≥ 40, Betweenness ≥ 0.007 and closeness ≥ 0.702, only 20 nodes and 178 edges were left (Fig. 4C). Therefore, these remained genes, IL6, INS, tumor necrosis factor (TNF), AKT1, vascular endothelial growth factor A (VEGFA), IGF1, TP53, IL1B, MMP9, JUN, LEP, CTNNB1, EGF, PTGS2, PPARG, CXCL8, IL10, CCL2, FOS and ESR1 were considered as the hub therapeutic targets of LWDH Pill against PMOP. Their specific information were listed in Table 2.

3.4. GO biological process enrichment analysis

GO enrichment analysis was performed on these 84 overlapping genes by using the Annotation, Visualization and Integrated Discovery database. Based on the filter of FDR < 0.01, a total of 240 GO items were obtained, including 196 BP terms, 13 cellular component terms and 31 molecular function terms (Fig. 5A). As BP played a dominant role, we further build a bubble diagram for the top 40 BP terms (Fig. 5B). The diagram showed that these 40 BP terms were mainly summarized into 5 categories, including inflammation, oxidative stress, angiogenesis, cell proliferation and cell apoptosis. The category of cell proliferation included positive regulation of gene expression (GO:0010628), positive regulation of transcription DNA-templated (GO:0045893), negative regulation of gene expression (GO:0010629), positive regulation of transcription from RNA polymerase II promoter (GO:0045944), negative regulation of cell proliferation (GO:0008285) and positive regulation of cell proliferation (GO:0008284). There are 5 BP terms in the category of inflammation, including response to lipopolysaccharide (GO:0032496), inflammatory response (GO:0006954), lipopolysaccharide-mediated signaling pathway (GO:0031663), response to glucocorticoid (GO:0051384) and immune response (GO:0006955). Regarding to oxidative stress, there are response to hypoxia (GO:0001666), positive regulation of nitric oxide biosynthetic process (GO:0045429), positive regulation of nitric-oxide synthase activity (GO:0051000) and

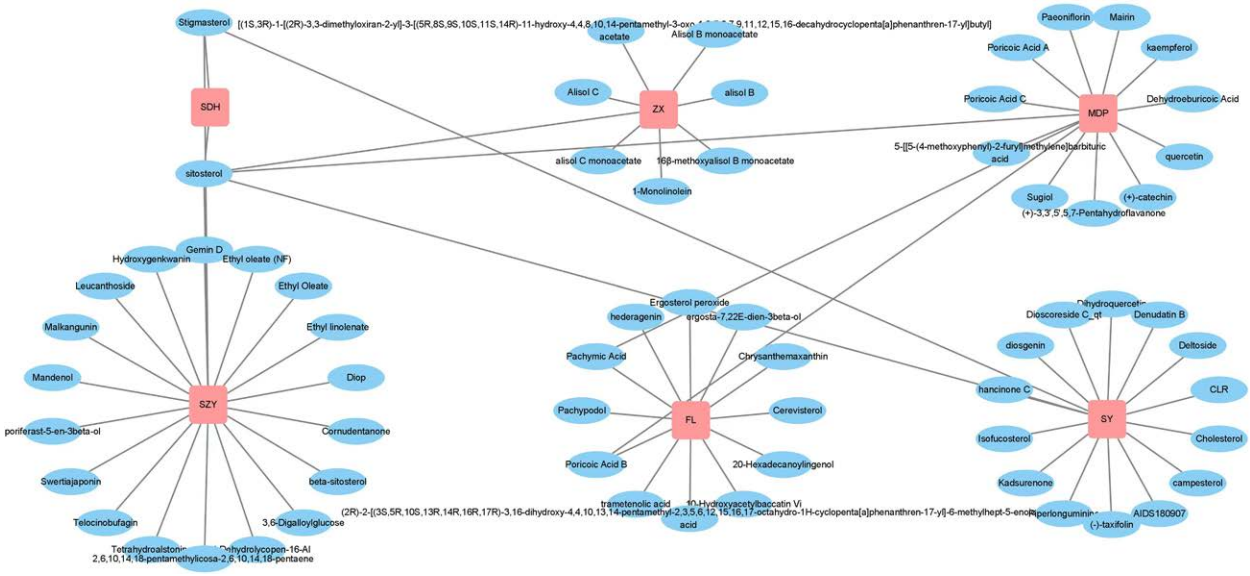

Figure 2. The network of Herb-Ingredient connection. The red square nodes represent herbs in LWDH Pill, and the blue circles represent active ingredients. The edges represent the direct relationship between them. LWDH = Liuwei Dihuang.

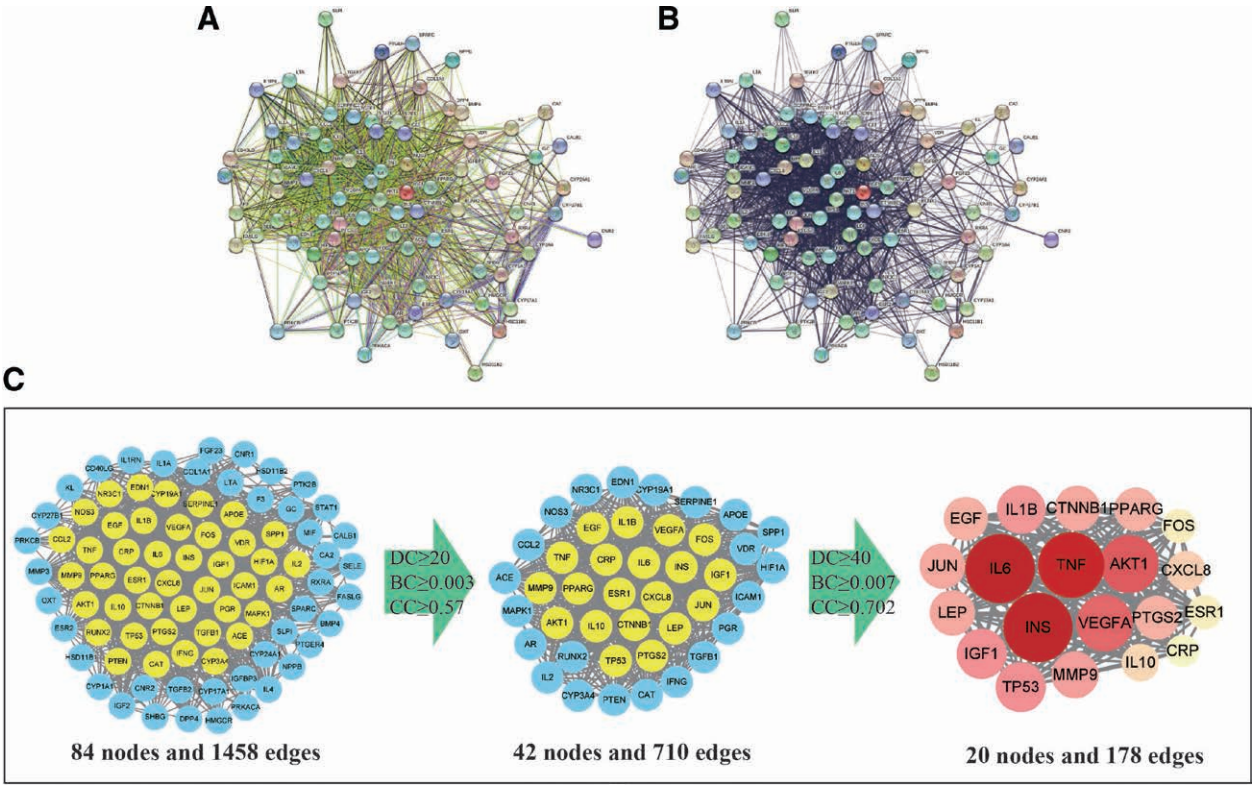

Figure 4. PPI network construction and screening. (A, B) PPI networks constructed using String database. Line colors indicate the type of interactive evidence. Line thickness indicates the confidence level of the supporting data. (C) The topological screening with (DC), (BC) and (CC) on PPI network. In the third image, the node with higher DC value represents more brilliant color and bigger size. BC = betweenness centrality, CC = closeness centrality, DC = degree centrality, PPI = protein-protein interaction.

cellular response to hypoxia (GO:0071456). In the aspect of angiogenesis, there were angiogenesis (GO:0001525), positive regulation of angiogenesis (GO:0045766) and positive regulation of endothelial cell proliferation (GO:0001938). The category of cell apoptosis included positive regulation of apoptotic process (GO:0043065) and negative regulation of apoptotic process (GO:0043066). These findings indicated that LWDH Pill exerted the anti-PMOP effects possibly through a multi-biological process synergetic way.

3.5. KEGG enrichment analysis

To further reveal the potential signaling pathways of LWDH Pill for PMOP treatment, we conducted KEGG enrichment analysis on the 84 overlapping genes. Based on the threshold of gene number ≥ 12, we screened out 32 signaling pathways (Fig. 6A), among which tumor necrosis factor (TNF) signaling pathway (hsa04668)[20] and interleukin-17 (IL-17) signaling pathway (hsa04657)[21] directly regulate inflammation; PI3K-Akt signaling pathway (hsa04151)[22] and mitogen-activated protein kinase (MAPK) signaling pathway (hsa04010)[23] are involved in cell proliferation and cell apoptosis; FoxO signaling pathway (hsa04068) plays an important role in oxidative stress[24]; hypoxia-inducible factors-1 (HIF-1) signaling pathway (hsa04066) controls angiogenesis.[25] Furthermore, a Targets-Pathways network was conducted to present the hub targets enriched in the above pathways (Fig. 6B).

4. Discussion

PMOP has become a public health disease worldwide with aging progression. The side effects caused by the long-term use of anti-osteoporosis drugs are calling for a more effective and safer treatment strategy. LWDH Pill has shown anti-osteoporosis effects both in animal model and PMOP patients.[7–10] In this study, we comprehensively explored the potential mechanisms of LWDH Pill through network pharmacology approach.

Based on the Herb-Ingredient network, we identified 2 key ingredients of LWDH Pill: beta-sitosterol and stigmasterol. Through a literature search, beta-sitosterol is found to alleviate ovariectomy-induced osteoporosis through promoting osteoblast proliferation and osteogenic activity.[26] Stigmasterol has an inhibition effect on osteoclastogenesis through anti-inflammation.[27,28] These active ingredients are the material foundations for the anti-PMOP effects of LWDH Pill.

After collecting the overlapping genes between LWDH Pill and PMOP in the Venn diagram, we identified a total of 84 potential therapeutic targets. PPI topological screening with Degree Centrality, Closeness Centrality and Betweenness Centrality further revealed 20 hub targets that were regarded as key molecular targets of LWDH Pill for PMOP treatment. According to the findings in GO and KEGG enrichment analyses on the 84 overlapping genes, we speculated that LWDH Pill treating PMOP was possibly associated with the regulation of several BP (cell proliferation, cell apoptosis, inflammation, oxidative stress and angiogenesis) and signaling pathways (TNF signaling pathway, IL-17 signaling pathway, FoxO signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, and HIF-1 signaling pathway).

4.1. Cell proliferation and apoptosis

Bone homeostasis requires dynamic balance between bone formation and bone resorption.[29] Osteoporosis occurs when osteoblast-mediated bone formation is slower than bone resorption directed by osteoclasts.[30] Thus, the abnormal proliferation and apoptosis of osteoblasts and osteoclasts directly result in osteoporosis. Epidermal growth factor (EGF), 1 of 20 hub overlapping

Table 2

Information of 20 hub target genes.

Uniprot ID	Gene Symbol	Description	Degree
P05231	IL6	Interleukin-6	72
P01308	INS	Insulin	72
P01375	TNF	Tumor necrosis factor	70
P31749	AKT1	RAC-alpha serine/threonine-protein kinase	65
P15692	VEGFA	Vascular endothelial growth factor A	64
P05019	IGF1	Insulin-like growth factor I	60
P04637	TP53	Cellular tumor antigen p53	60
P01584	IL1B	Interleukin-1 beta	59
P14780	MMP9	Matrix metalloproteinase-9	58
P05412	JUN	Transcription factor AP-1	57
P41159	LEP	Leptin	57
P35222	CTNNB1	Catenin beta-1	57
P01133	EGF	Pro-epidermal growth factor	56
P35354	PTGS2	Prostaglandin G/H synthase 2	56
P37231	PPARG	Peroxisome proliferator-activated receptor gamma	56
P10145	CXCL8	Interleukin-8	53
P22301	IL10	Interleukin-10	52
P13500	CCL2	C-C motif chemokine 2	51
P01100	FOS	Protein c-Fos	50
P03372	ESR1	Estrogen receptor	50

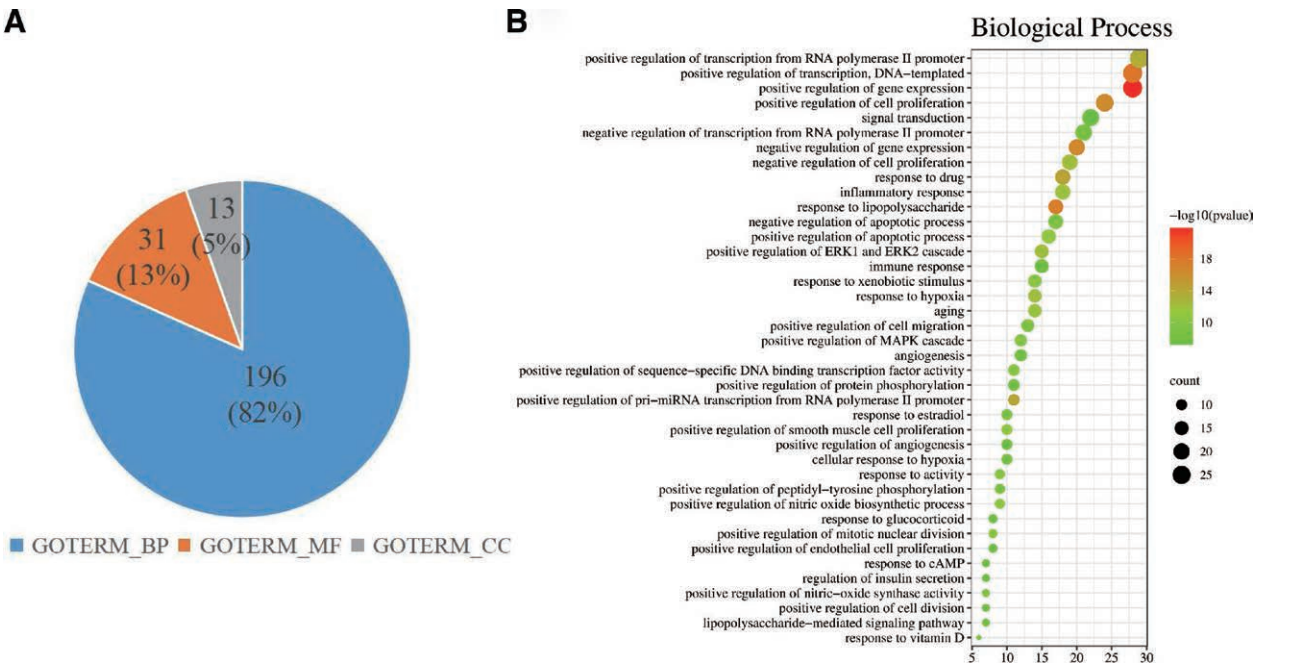

Figure 5. GO enrichment analysis of the 84 overlapping genes. (A) Three types of GO items in DAVID database (Blue, GOTRIM_BP: biological process. Yellow, GOTRIM_CC: cellular component. Gray, GOTRIM_MF: molecular function). (B) The bubble diagram of the top 40 BP items. DAVID = the Annotation, Visualization and Integrated Discovery.

targets, can activate PI3K-AKT pathway to promote osteoblast proliferation for osteogenesis.[31] It is further reported that OVX-induced osteoporosis in rats can be treated through promoting PI3K-AKT pathway-mediated bone formation.[32] In contrast, MAPK signaling pathway contains 10 hub overlapping target genes (EGF, AKT1, FOS, IGF1, IL1B, INS, JUN, TNF, TP53 and VEGFA) and regulates osteoclastic bone resorption.[33] The down-regulation of MAPK signaling can attenuate postmenopausal bone loss by inhibiting osteoclast proliferation.[34] Therefore, it is reasonable to believe, cell proliferation regulated by PI3K-AKT pathway and MAPK pathway is a part mechanism of LWDH Pill against PMOP.

The balance between osteoblast proliferation and apoptosis affects bone formation homeostasis, thereby osteoblast over apoptosis is a critical pathological mechanism of PMOP. It has been reported that inhibition of PI3K/AKT signaling pathway could promote osteoblast apoptosis in the OVX mice.[35] The Targets-Pathways network revealed 7 hub targets (EGF, AKT1, IGF1, IL6, INS, TP53 and VEGFA) enriched in PI3K/AKT signaling pathway, indicating that PI3K/AKT signaling inhibiting osteoblast apoptosis is involved in the therapeutic mechanism of LWDH Pill. IL-1A and IL1B were overlapping targets between LWDH Pill and PMOP, and were involved in MAPK signaling pathway. Previous study revealed that IL-1 could induce osteoblast apoptosis through activating MAPK pathways.[36] Thus, MAPK signaling pathway may be potential pharmacological mechanism when LWDH Pill treats PMOP.

4.2. Inflammation

Inflammation is 1 of most important pathological factors that contributes to the occurrence and development of PMOP. Most

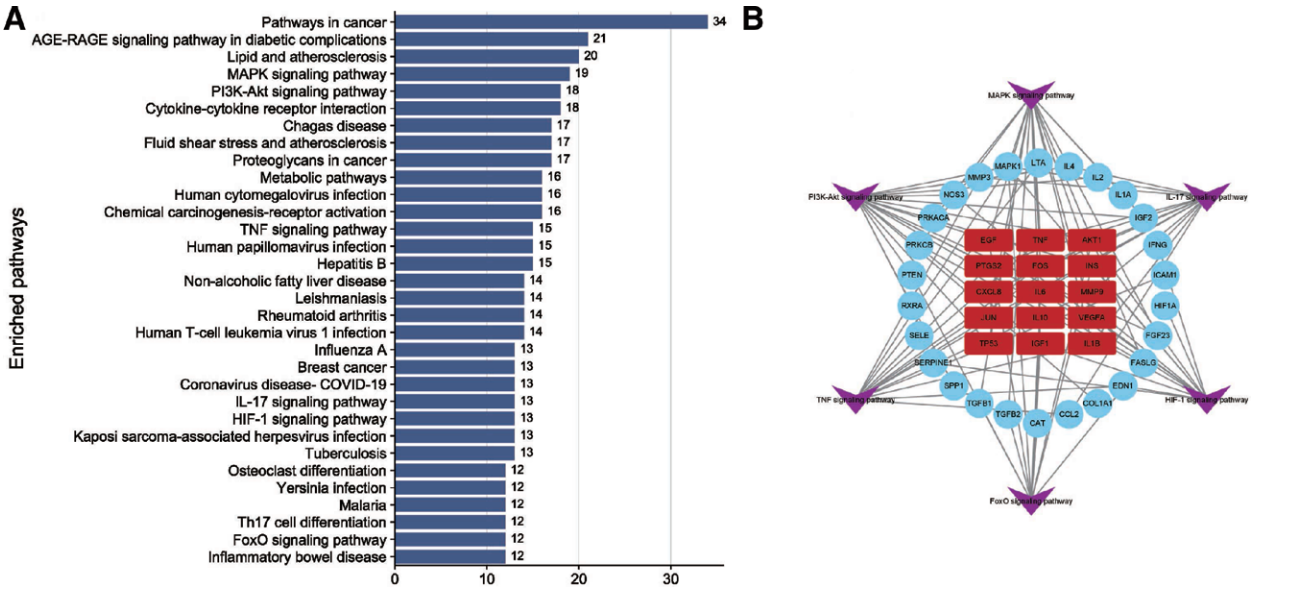

Figure 6. KEGG enrichment analysis and Targets-Pathways network. (A) Details of the top 32 pathways in KEGG database. (B) Network between 6 key pathways and target genes (red rectangles represent hub targets). KEGG = Kyoto encyclopedia of genes and genomes.

of pro-inflammatory cytokines can directly influence bone resorption though regulating osteoclast activity.[37] TNF synthesized by T-lymphocytes can accelerate bone loss through 2 mechanisms which are promoting osteoclastogenesis and inhibiting apoptosis of osteoclasts.[38] Interleukin-1 (IL-1), a multifunctional cytokine, is considered as "osteoclast activating factor."[39] It signals on osteoclast lineage cells to stimulate osteoclastogenesis and resorptive capacity in receptor activator of nuclear factors κB ligand-dependent way.[40] IL-1 can also interact with TNF to protect osteoclast apoptosis. Interleukin-6 (IL-6) is highly increased after estrogen loss[41] and closely related to low bone mineral density.[42] IL-6 stimulates bone resorption by activating receptor activator of nuclear factors κB ligand/osteoprotegerin pathway.[43] Based on hub Target-Pathway network, TNF, IL1B and IL-6 are enriched in TNF signaling pathway and IL-17 signaling pathway. Hence, the anti-PMOP effects of LWDH Pill may possibly be associated with the inhibition of inflammation-induced bone resorption.

4.3. Oxidative stress

Oxidative stress is associated with PMOP, as excessive reactive oxygen species are able to exert oxidative damage to mitochondria and DNA in osteoblast and osteoclast.[44] Currently, inhibition of oxidative stress is regarded as a therapeutic target for treating PMOP. Many studies have revealed the regulating effects of FoxO signaling pathway on oxidative stress. FoxO1 was found to promote osteoblast proliferation by resistance to oxidative stress.[45] Loss function of FoxO signaling increased osteoclast-mediated bone resorption by releasing H_2O_2.[46] There are 12 hub targets (EGF, AKT1, IGF1, FASLG, IL6, INS, MAPK1 and PTEN) were enriched, indicating that inhibition of oxidative stress regulated by FoxO signaling pathway is a important mechanism of LWDH Pill against PMOP.

4.4. Angiogenesis

Bone is a highly vascularized tissue with a wide network of capillaries and blood vessels. New bone formation needs a spatial and temporal involvement of vascularization to provide essential nutrients, oxygen and growth factors.[47] More remarkably, angiogenesis directly promotes bone formation, which is named angiogenesis-osteogenesis coupling.[48,49] VEGFA, a major driver of angiogenesis, can activate a series of well-orchestral vascularization processes including endothelial cell proliferation, migration, sprouting vessel pruning and anastomosis.[50] HIF-1α is a transcription factor that can bind to VEGF gene promoter and induce VEGF gene expression.[51] KEGG enrichment analysis and Targets-Pathways network revealed that VEGFA and HIF-1 pathway were both identified in the anti-PMOP event of LWDH Pill, and VEGFA was contained in HIF-1 pathway. Thus, promotion of angiogenesis regulated by HIF-1 pathway may be a potential mechanism of LWDH Pill in treatment of PMOP.

4.5. Limitations

There are several limitations in the present study. First and foremost, network pharmacology is a discipline of calculation and prediction, which is bound to have some false positives in ingredients, BP and signaling pathways. Secondly, we screened 3 databases to obtain active ingredients and 2 databases for disease-related targets, while there still might leave out some of ingredients and targets. Finally, in vivo and in vitro experiments need to be performed further to verify these predicted BP and signaling pathways.

5. Conclusion

By utilizing network pharmacology, we explored the potential therapeutic targets of LWDH Pill, and investigated its underlying mechanisms against PMOP. As we discovered, it might be based on 3 key BP: regulation of cell proliferation and apoptosis, promotion of angiogenesis, inhibition of inflammation and oxidative stress. KEGG pathway analysis revealed the pathways involved in anti-PMOP event of LWDH Pill as follows: TNF signaling pathway, IL-17 signaling pathway, FoxO signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, and HIF-1 signaling pathway. Therefore, it can be concluded that the pharmacological mechanism of LWDH Pill against PMOP is a direct or indirect synergy way of multi-targets and multi-pathways. Although it still needs further experimental validation to determine their exact pharmacological mechanism, our results provide promising directions for future research.

All our main data used to support the findings of this study have been deposited by the corresponding author.

Author contributions

Conceptualization: Qingchan Wang, Chenjie Xia, Danqing Fu.
Data curation: Qingchan Wang, Chenjie Xia, Danqing Fu.
Formal analysis: Ping Huang, Danqing Fu.
Funding acquisition: Chenjie Xia, Danqing Fu.
Methodology: Qingchan Wang, Ping Huang, Danqing Fu.
Project administration: Qingchan Wang, Ping Huang, Danqing Fu.
Software: Qingchan Wang, Ping Huang, Chenjie Xia.
Supervision: Ping Huang.
Validation: Qingchan Wang, Ping Huang, Chenjie Xia, Danqing Fu.
Visualization: Qingchan Wang, Chenjie Xia, Danqing Fu.
Writing – original draft: Chenjie Xia.
Writing – review & editing: Qingchan Wang, Chenjie Xia, Danqing Fu.

References

[1] Compston JE, McClung MR, Leslie WD. Osteoporosis. Lancet. 2019;393:364–76.
[2] Wright NC, Looker AC, Saag KG, et al. The recent prevalence of osteoporosis and low bone mass in the United States based on bone mineral density at the femoral neck or lumbar spine. J Bone Miner Res. 2014;29:2520–6.
[3] Langdahl BL. Overview of treatment approaches to osteoporosis. Br J Pharmacol. 2020;2:1–6.
[4] Khosla S, Hofbauer LC. Osteoporosis treatment: recent developments and ongoing challenges. Lancet Diabetes Endocrinol. 2017;5:898–907.
[5] Liu Y, Liu JP, Xia Y. Chinese herbal medicines for treating osteoporosis. Cochrane Database Syst Rev. 2014;3:CD005467.
[6] Ge JR, Xie LH, Chen J, et al. Liuwei Dihuang pill treats postmenopausal osteoporosis with Shen (kidney) Yin deficiency via Janus Kinase/signal transducer and activator of transcription signal pathway by up-regulating Cardiotrophin-like cytokine factor 1 expression. Chin J Integr Med. 2018;24:415–22.
[7] Liu Y, Wang P, Shi X, et al. Liuwei Dihuang secoction for primary osteoporosis: a protocol for a systematic review and meta-analysis. Medicine (Baltim). 2019;98:e15282.
[8] Xia B, Xu B, Sun Y, et al. The effects of Liuwei Dihuang on canonical Wnt/β-catenin signaling pathway in osteoporosis. J Ethnopharmacol. 2014;153:133–41.
[9] Jing Y, Cai D, Chen Q, et al. Liuwei Dihuang soft capsules attenuates endothelial cell apoptosis to prevent atherosclerosis through GPR30-mediated regulation in ovariectomized ApoE-deficient mice. J Ethnopharmacol. 2017;208:185–98.
[10] Xu F, Gao F. Liuwei Dihuang pill cures postmenopausal osteoporosis with kidney-Yin deficiency: potential therapeutic targets identified based on gene expression profiling. Medicine (Baltim). 2018;97:e11659.
[11] Ke C, Chen Y, Wang Y, et al. Exploring the mechanism of Bushenhuoxue formula acting on postmenopausal osteoporosis via network pharmacology and experimental validation. Sci Rep. Under Review. doi: 10.21203/rs.3.rs-156672/v1.
[12] Hopkins AL. Network pharmacology. Nat Biotechnol. 2007;25:1110–1.
[13] Li S, Zhang B. Traditional Chinese medicine network pharmacology: theory, methodology and application. Chin J Nat Med. 2013;11:110–20.
[14] Zhang R, Zhu X, Bai H, et al. Network pharmacology databases for traditional Chinese medicine: review and assessment. Front Pharmacol. 2019;10:123.
[15] Zhu N, Hou J. Exploring the mechanism of action Xianlingubao Prescription in the treatment of osteoporosis by network pharmacology. Comput Biol Chem. 2020;85:107240.
[16] Han J, Wan M, Ma Z, et al. Prediction of targets of curculigoside A in osteoporosis and rheumatoid arthritis using network pharmacology and experimental verification. Drug Des Devel Ther. 2020;14:5235–50.
[17] Missiuro PV, Liu K, Zou L, et al. Information flow analysis of interactome networks. PLoS Comput Biol. 2009;5:e1000350.
[18] Tang Y, Li M, Wang J, et al. CytoNCA: a cytoscape plugin for centrality analysis and evaluation of protein interaction networks. Biosystems. 2015;127:67–72.
[19] Raman K, Damaraju N, Joshi GK. The organisational structure of protein networks: revisiting the centrality-lethality hypothesis. Syst Synth Biol 2014;8:73–81.
[20] Presicce P, Cappelletti M, Senthamaraikannan P, et al. TNF-signaling modulates neutrophil-mediated immunity at the Feto-maternal interface during LPS-induced intrauterine inflammation. Front Immunol. 2020;11:558.
[21] Park MJ, Moon SJ, Lee EJ, et al. IL-1-IL-17 signaling axis contributes to fibrosis and inflammation in two different murine models of systemic sclerosis. Front Immunol. 2018;9:1611.
[22] Bishnupuri KS, Alvarado DM, Khouri AN, et al. IDO1 and kynurenine pathway metabolites activate PI3K-Akt signaling in the neoplastic colon epithelium to promote cancer cell proliferation and inhibit apoptosis. Cancer Res. 2019;79:1138–50.
[23] Sun Y, Liu WZ, Liu T, et al. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J Recept Signal Transduct Res. 2015;35:600–4.
[24] Almeida M, Porter RM. Sirtuins and FoxOs in osteoporosis and osteoarthritis. Bone. 2019;121:284–92.
[25] Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol. 2006;70:1469–80.
[26] Chauhan S, Sharma A, Upadhyay NK, et al. In-vitro osteoblast proliferation and in-vivo anti-osteoporotic activity of Bombax ceiba with quantification of Lupeol, gallic acid and β-sitosterol by HPTLC and HPLC. BMC Complement Altern Med. 2018;18:233.
[27] Das N, Bhattacharya A, Kumar Mandal S, et al. Ichnocarpus frutescens (L.) R. Br. root derived phyto-steroids defends inflammation and algesia by pulling down the pro-inflammatory and nociceptive pain mediators: An in-vitro and in-vivo appraisal. Steroids. 2018;139:18–27.
[28] Kim EH, Jo CS, Ryu SY, et al. Anti-osteoclastogenic diacetylenic components of Dendropanax morbifera. Arch Pharm Res. 2018;41:506–12.
[29] Guo L, Chen K, Yuan J, et al. Estrogen inhibits osteoclasts formation and bone resorption via microRNA-27a targeting PPARγ and APC. J Cell Physiol. 2018;234:581–94.
[30] Kiernan J, Davies JE, Stanford WL. Concise review: musculoskeletal stem cells to treat age-related osteoporosis. Stem Cells Transl Med. 2017;6:1930–9.
[31] Xi JC, Zang HY, Guo LX, et al. The PI3K/AKT cell signaling pathway is involved in regulation of osteoporosis. J Recept Signal Transduct Res. 2015;35:640–5.
[32] Zheng D, Cui C, Yu M, et al. Coenzyme Q10 promotes osteoblast proliferation and differentiation and protects against ovariectomy-induced osteoporosis. Mol Med Rep. 2018;17:400–7.
[33] Cong Q, Jia H, Li P, et al. p38α MAPK regulates proliferation and differentiation of osteoclast progenitors and bone remodeling in an aging-dependent manner. Sci Rep. 2017;7:45964.
[34] Wang W, Bai J, Zhang W, et al. Protective Effects of Punicalagin on Osteoporosis by Inhibiting Osteoclastogenesis and Inflammation via the NF-κB and MAPK Pathways. Front Pharmacol. 2020;11:696.
[35] Zhang Y, Cao X, Li P, et al. PSMC6 promotes osteoblast apoptosis through inhibiting PI3K/AKT signaling pathway activation in ovariectomy-induced osteoporosis mouse model. J Cell Physiol. 2020;235:5511–24.
[36] Guo C, Yang XG, Wang F, et al. IL-1α induces apoptosis and inhibits the osteoblast differentiation of MC3T3-E1 cells through the JNK and p38 MAPK pathways. Int J Mol Med. 2016;38:319–27.
[37] Briot K, Geusens P, Em Bultink I, et al. Inflammatory diseases and bone fragility. Osteoporos Int. 2017;28:3301–14.
[38] Weitzmann MN, Pacifici R. The role of T lymphocytes in bone metabolism. Immunol Rev. 2005;208:154–68.
[39] De Martinis M, Ginaldi L, Sirufo MM, et al. Alarmins in Osteoporosis, RAGE, IL-1, and IL-33 Pathways: A Literature Review. Medicina (Kaunas) 2020;56:1–10.
[40] Kim JH, Jin HM, Kim K, et al. The mechanism of osteoclast differentiation induced by IL-1. J Immunol. 2009;183:1862–70.
[41] Yan L, Hu R, Tu S, et al. Meta-analysis of association between IL-6 -634C/G polymorphism and osteoporosis. Genet Mol Res. 2015;14:19225–32.
[42] Wang Z, Yang Y, He M, et al. Association between interleukin-6 gene polymorphisms and bone mineral density: a meta-analysis. Genet Test Mol Biomarkers. 2013;17:898–909.
[43] Silva R, Sousa-Pereira AP, Lucisano MP, et al. Alendronate inhibits osteocyte apoptosis and inflammation via IL-6, inhibiting bone resorption in periapical lesions of ovariectomized rats. Int Endod J. 2020;53:84–96.

[44] Zhou Q, Zhu L, Zhang D, et al. Oxidative Stress-Related Biomarkers in Postmenopausal Osteoporosis: A Systematic Review and Meta-Analyses. Dis Markers. 2016;12:1–8.
[45] Rached MT, Kode A, Xu L, et al. FoxO1 is a positive regulator of bone formation by favoring protein synthesis and resistance to oxidative stress in osteoblasts. Cell Metab. 2010;11:147–60.
[46] Bartell SM, Kim HN, Ambrogini E, et al. FoxO proteins restrain osteoclastogenesis and bone resorption by attenuating H2O2 accumulation. Nat Commun. 2014;5:3773–9.
[47] Taufik NH, Tulaar A, Moesbar N, et al. The Effect of Isometric Exercise Plantar Flexor on Osteoblast Activity Measured by Bone Specific Alkaline Phosphatase and Callus Formation in a Patient Post Open Reduction Internal Fixation with Non-articular Tibia Fracture. Open Access Maced J Med Sci 2019;7:3409–15.
[48] Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature 2014;507:323–8.
[49] Xie H, Cui Z, Wang L, et al. PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis. Nat Med. 2014;20:1270–8.
[50] Herbert SP, Stainier DY. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nat Rev Mol Cell Biol. 2011;12:551–64.
[51] Ahluwalia A, Tarnawski AS. Critical role of hypoxia sensor--HIF-1α in VEGF gene activation. Implications for angiogenesis and tissue injury healing. Curr Med Chem. 2012;19:90–7.